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14. ABSTRACT

In this second annual report, we present our progress on two different areas, protein production and crystallization. We are working on expression and purification of Yop proteins and their complexes. We have made progress in purifying YopB:SycD complex. We are also purifying Yop B, D and E complex to understand the translocation machinery.

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Structural Studies on Toxins and Virulence Factors of *Yersinia pestis*

Progress Report for the Period ending June 2005

Introduction

The overall goal of this project is to determine the three-dimensional structures of proteins and virulence factors from *Yersinia pestis* to understand the mechanism of translocation of effector proteins. *Yersinia pestis* is responsible for the plague and is a potential and emerging biowarfare threat. This organism has evolved a powerful method of delivering effectors into cells of the host immune system. The effectors act to completely circumvent the immune system. Understanding the molecular mechanism of this system is important to get an insight into the process of phagocytosis and inflammation. The effector proteins are secreted into the cells by a type III secretion system which involves a number of proteins. Some of them are actively involved in the delivery system and others are required for pore formation. Information derived from this study will help in developing vaccines and therapeutics for the plague. It will also help in identifying genetically engineered threats. The goal is to study the three-dimensional structures of Yop proteins and their corresponding chaperones. By studying the three-dimensional structures of effector proteins we can get an insight into the mechanism of toxification of the cells which would eventually lead to design of inhibitors to block the toxicity.

Body

Purification and crystallization of YopH:

YopH is a key translocated effector of *Y. pestis* that exhibits tyrosine phosphatase activity. The 50 kDa YopH protein is organized into two modular domains separated by a linker region. The N terminal domain has phosphopeptide binding activity and functions as a substrate-targeting domain for the C-terminal catalytic domain. The two separate domains of YopH have been crystallized and their structures solved (1,2). However, the structure of the native protein has not been determined. This is likely due to the fact that the native protein does not form crystals because of flexibility between the two domains. We believe that determining the structure of the native protein is

important, as it will provide clues into potential functional interactions between the two domains. As part of this project we have undertaken studies to obtain the structure of the intact YopH protein (468 aa). A catalytically inactive form of YopH protein has been overexpressed with a C-terminal 6XHis tag in *E. coli* and purified by nickel affinity chromatography. The yield is typically 40 mg of protein per liter of culture volume.

Crystallization trials have been initiated using samples of the purified protein and still in progress. The failure to obtain crystallization condition so far may be because of the flexibility of the protein. We have developed a strategy to overcome the flexibility between the two domains using a synthetic peptide substrate with two phosphorylation sites that we hope will be simultaneously engaged by each domain, thereby imparting a rigid conformation to the protein. Since not much success has been achieved in this, we are now trying to change the linker length in order for the peptide to bind to both the phosphorylation sites simultaneously.

YopH-peptide substrate complex:

YopH protein binds to focal adhesion proteins such as Crk-associated substrate, p130 (p130Cas) of epithelial cells. The p130Cas is one of the proteins with many phosphorylated tyrosines that are critical for many functions like cell-cell interaction, metabolism and immune response. While the N-terminal domain is able to recognize the substrate protein, the C-terminal domain dephosphorylates it. Both the domains have phosphorylated tyrosine binding sites and the YopH binds to p130Cas in a phophoryl-dependent manner. A second p130Cas binding site has been identified recently in the catalytic domain of YopH and this site is responsible along with the N-terminal site for the protein-substrate binding. In the process of YopH-p130Cas complex formation and dephosphorylation, the proteins interact at more than one site and both domains are involved. This implies that the substrate binds to the intact YopH holding the two domains intact. To explore this possibility we are trying to crystallize the intact YopH in complex with peptides that has two phosphorylated tyrosines. Two synthetic peptide substrates based on a natural substrate (Cas) of YopH have been synthesized. The sequences of these peptides are shown below, with phosphorylated tyrosines (pY) shown in bold:

Cas peptide (342-371): GSQDI**pY**DVPPVRGLLPNQYGQEV**pY**DTPPMA

Cas peptide (258-287): PATDL**pY**QVPPGPGSPAQDI**pY**QVPPSAGTGH

Crystallization trials of YopH with each of these peptides are in progress. These peptides will be individually mixed with purified inactive YopH and crystallization studies will be initiated. If the substrate binding sites of the N-terminal domain and the catalytic domain are in close proximity in the native protein, we anticipate that the peptides will form a bridge between the two domains and create a rigid conformation in the protein that will facilitate crystallization. Work is in progress.

Preliminary structural characterization of YopB, YopD and YopK:

Delivery of secreted effectors such as YopH into host cells infected with *Y. pestis* is controlled by a set of secreted "translocator" proteins. Three proteins are required for the translocation process, LcrV, YopB and YopD. A fourth protein, YopK, appears to function as a negative regulator of translocation. The structure of a mutant form of LcrV has recently been determined (3). However, the structures of YopB, YopD and YopK have not been determined. YopB (42 kDa) and YopD (33 kDa) contain predicted transmembrane domains and are thought to form a channel in the host cell membrane. YopK (21 kDa) does not have any recognizable features, and its function remains mysterious. Attempts to overexpress recombinant forms of YopB and YopD in *E. coli* have met with no success, due to their hydrophobic characteristics. As an alternative approach, we have engineered *Yersinia* to secrete these proteins into growth media, and we have investigated the possibility that the secreted forms of these proteins could be used for structural determinations. Currently we are working with relatively small volumes of culture (less than 100 ml) and proteins secreted into *Yersinia* growth media are concentrated by filtration and analyzed by native polyacrylamide gel electrophoresis. Although yields of protein are small (e.g. 10 micrograms) this procedure does provide enough protein for initial studies. The results of these experiments showed that all three proteins, YopB, YopD and YopK, were secreted as soluble proteins. However, all three proteins migrated as multimeric forms on the gels. YopB and YopD formed multimeric ladders of bands on the gels, and YopK ran as a broad band at a very high molecular

weight. Thus, although the secreted forms of YopB, YopD and YopK are soluble, the multimeric forms of these proteins will make protein crystallization and X-ray determination challenging. Preliminary results indicate that YopB and YopD are forming homo- and hetero-oligomers. We plan additional studies in Year 2 (see below) to determine if the hetero-oligomeric forms of YopB and YopD are suitable for crystallization studies. However, we are also trying to express YopB and YopD individually as membrane proteins.

YopB-YopD complex:

Preliminary studies indicate that YopB and YopD are secreted into growth media by *Yersinia* in a soluble form. These proteins appear to be forming homo- and hetero-oligomeric complexes. It is possible that the hydrophobic regions of these proteins are buried in the oligomers, which allows them to be soluble in aqueous solutions. Although the homo-oligomeric forms of these proteins may not be suitable for crystallization, we anticipate that the hetero-oligomeric complexes may represent distinct species that could be isolated in pure form and used for crystallization studies. To investigate this possibility, we will purify a secreted form of YopB that contains an N-terminal 6X his tag. We have confirmed that this tagged form of YopB retains full biological activity. The purified YopB will be run on native gels to determine the percentage of the protein that is in a homo- or hetero-oligomeric form. If we are able to identify a specific YopB-YopD complex on native gels, we will then attempt to obtain sufficient amounts of this complex for structural studies. So far not much success has been achieved towards this.

Co-expression of YopB and SycD in E. coli:

We have cloned YopB and YopD in pET vector. However, expression of these proteins in *E.coli* is still a challenge. The quantity that we obtain is not enough for our crystallization trials. In addition to getting YopB:YopD complex by co expression, we are also trying another technique. SycD is an intrabacterial protein chaperone. It has been shown that SycD binds to YopB. We are now attempting to co-express YopB with SycD. For this we have cloned YopB with and without histag and SycD with and without histag. When they are expressed together neither of them is toxic to the cell unlike when YopB alone is expressed.

Progress in expression and purification of YopB:sycD complex:

As described earlier we are working with two systems for co-expression of YopB and SycD (A) pet28A-YopB - SycD and (B) pet28A-SycD - YopB. These two pairs of vectors were introduced into Turner (DE3) cells for expression. We followed the traditional IPTG induction method and the recently developed auto-induction method by Studier (4). With B we found that SycD expresses much more than YopB in IPTG induction method. But when A was used, both proteins were not expressed well with IPTG induction. Next we tried to express the two by auto induction method. In this method with A both YopB and SycD express well and in a stoichiometric ratio whereas it did not work well with B. In view of this we are using co-expression of pet28a-YopB-SycD by auto induction method. Here again we are optimizing the condition for expression by changing parameters in auto-induction (e.g. – addition of metal mix).

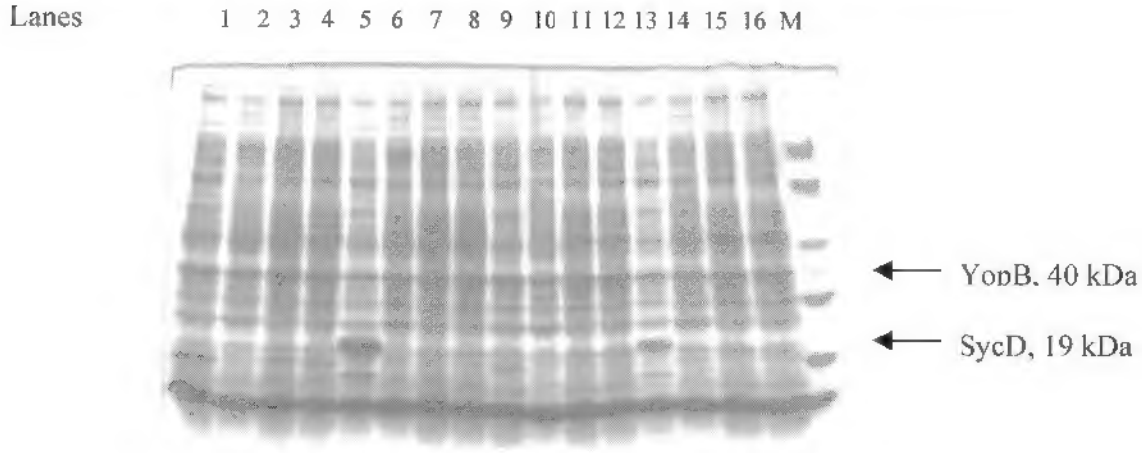

Figure 1: SDS-Page gel with various trials of co-expression of YopB and SycD. Lanes 1 4 represent the total lysate from A by IPTG induction, MDG medium grown cell and with auto-induction with and without metal mix. Lane 5 to 8 is total lysate with B in the same order. In lane 5 it is evident that the expression of SycD is much more than YopB whereas neither YopB nor SycD expresses well in A (lane 1). Lane 9 to 12 represent the supernatant of A in the same order while lanes 13 to 16 represent that of B in the same order. Lane 17 corresponds to the molecular weight marker. Lanes 11 and 12 are of interest in this discussion. In both we find that both the proteins are expressed equally well. Addition of metal mix has no effect on expression.

This protein was further purified using Ni affinity column and tested and identified with western blot using anti-YopB. The western blot is presented below.

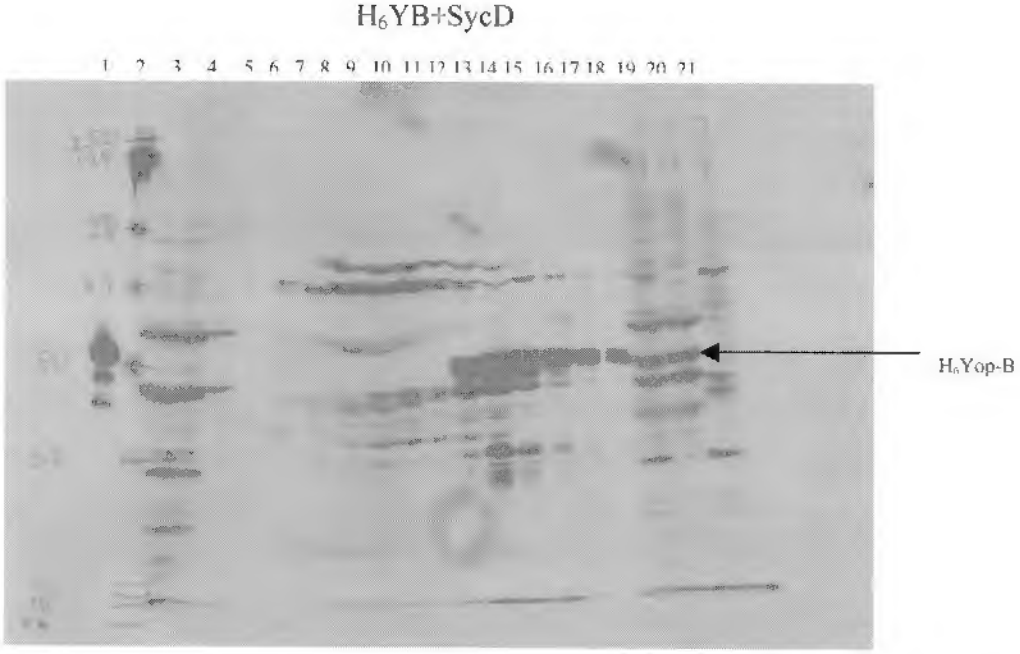

Figure 2: Lane 1. Control Molecular marker; 2. Supernatant; 3. Flow through; 4. Buffer wash; 5. 1mM imidazole wash; 6. 5mM imidazole; 7. 10mM; 8. 20mM (I); 9. 20mM (II); 10. 20mM (III); 11. 50mM; 12. 100mM (I); 13. 100mM (II); 14. 250mM (I); 16. 250mM (II); 17. 250mM (III); 18. 500mM; Total Lysate; 20. Supernatant; 21. Pellet

Chemiluminescence at lanes 13 to19 shows the presence H_6Yop-B at 40 kDa .

Further work is in progress.

Expression of Yop B, D and E complex:

Yersinia pestis is the agent of bubonic and pneumonic plague. Y. pestis is classified as a Category A pathogen because it has suitable characteristics for biological weapon development. Y. pestis utilizes a type III secretion system to deliver a set of protein toxins known as Yops directly into human cells. These toxins are delivered across the host cell membrane by a bacterial surface structure called the Yop translocon. The translocon is thought to be composed of at least three proteins: LcrV, YopD and YopB. LcrV, YopD and YopB are believed to form a channel in the host cell membrane through which the Yops such as YopE are delivered. However, the structure of the translocon has never been solved.

As an attempt toward determining the structure of the translocon, we engineered *Y. pseudotuberculosis* (a less virulent relative of *Y. pestis*) to secrete His-tagged YopB as well as native YopD and native YopE into bacteria growth media. Using this approach, we have been able to purify sufficient amounts of a His-YopB/YopD/YopE complex.

The figure below shows nickel affinity purification of a complex of His-YopB, yopD and YopE from supernatants of Yersinia cultures.

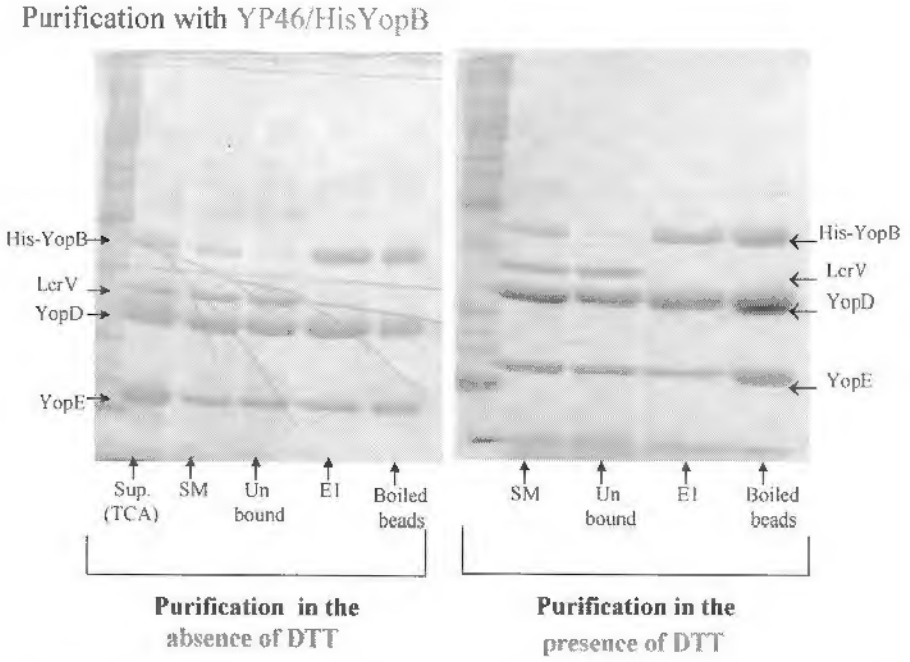

Figure 3. Sup. Stands for supernatant, SM for starting material (after concentration of the sup by ultrafiltration), unbound is what does not bind to the nickel column E1 is imidazole elution fraction 1, and 'boiled beads' is what does not elute of the column.

As the results show, we have been able to purify small amounts of a soluble complex of His-YopB, YopD and YopE in the E1 fraction. Each purification yields about 100 micrograms of protein. We have carried out the purification in the presence and absence of DTT and get similar results.

The results of native gels suggest that the complex is a mixture of oligomers. It seems that if a single species of the complex could be generated, perhaps using limited proteolysis would allow crystallization trials. We will also try and remove the His tag to

see if that helps reduce multimerization. At the same time, we will work toward increasing yields. Work is in progress.

Expression and Crystallization of LcrV:

LcrV was expressed in BL21 cells. The protein was expressed with Se labeled methionines that will be useful for the structure determination. Protein expression and purification were optimized, and 60mg of homogeneous protein was obtained per litter of culture. Purified protein was used for crystallization. Since the protein is highly soluble, 10 – 40 mg/ml protein concentration was used for crystallization trials. The protein crystallization attempts showed little clue of any crystallization conditions. In the meantime the crystal structure of LcrV triple mutant has been reported (3). It is perceived that the wild-type protein is recalcitrant to crystallization.

A mutation study on LcrV explains coiled-coil interaction of LcrV with LcrG (5). This paper identified the coiled-coil region for both the proteins of LcrV and LcrG. The coiled-coil region identified in LcrG is D7 through K28 (DEYDKTLKQAELAIADSDHRAK) and this region is presumed to interact with LcrV to form a complex. This 22aa peptide of LcrG was purchased and the complex of LcrV with this peptide has been made. Crystallization of this complex also has been tried. Complex with various molar ratios of LcrV and LcrG-peptide has been made and crystallization is in progress.

In the meantime optimizing the expression protocol of LcrG protein is in progress. Once we get LcrG protein we plan to make LcrV-LcrG complex. This will be more effective and useful for the project.

Crystallization of SycD:

SycD is the intrabacterial chaperone of YopD that directs the translocation of the secreted Yop effector proteins across the target membrane. Crystallization of this protein, SycD is also in progress. Expression and purification of this protein is successful and the initial crystallization trials are in progress.

Key Research Accomplishments: None yet.

Reportable outcomes

None as of now.

Conclusions

We have crossed one major hurdle of producing YopB in enough quantities for crystallization. We have achieved partial success in purifying Yop B, D and E complex.

Plans for the next year:

We will continue and complete the work in progress.

Personnel in the Project

1. S. Swaminathan (PI)	Scientist	20% effort
2. S. Eswaramoorthy	Associate Scientist	30% effort
3. L. Damodharan	Research Associate	100% effort

Sub-contract to State University of New York at Stony Brook

1. J. Bliska	Professor	10% effort
2. M. Ivanov	Technician	100% effort

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